APPLICATIONS OF MICELLAR ENZYMOLOGY TO CLEAN COAL TECHNOLOGY

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US/DOE Patent Clearance is not required prior to the publication of this document.

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Section 1

INTRODUCTION

1.1 Program Overview

Full implementation of coal fuel sources will require more effective methods of providing "clean coal" as a fuel source. Methods must be developed to reduce the sulfur content of coal which significantly contributes to environmental pollution. This project is designed to develop methods for pre-combustion coal remediation by implementing recent advances in enzyme biochemistry. The novel approach of this study is incorporation of hydrophilic oxidative enzymes in reverse micelles in an organic solvent. Enzymes from commercial sources or microbial extracts are being investigated for their capacity to remove organic sulfur from coal by oxidation of the sulfur groups, splitting of C-S bonds and loss of sulfur as sulfuric acid. Dibenzothiophene (DBT) and ethylphenylsulfide (EPS) are serving as models of organic sulfur-containing components of coal in initial studies.

A goal of this project is to define a reverse micelle system that optimizes the catalytic activity of enzymes toward desulfurization of model compounds and ultimately coal samples. Studies by several groups (Martinek et al., 1981; Kabanov *et al.*, 1988; Martinek, 1989; Verhaert *et al.*, 1990) have shown that the surfactant AOT over a broad concentration range in organic solvents produces micelles, comparatively uniform in diameter, which incorporate hydrophilic enzymes. The activity (kcat) of certain enzymes in this system is higher than in aqueous solution. This surfactant is therefore being examined as a vehicle for enhancement of sulfoxidation reactions.

1.2 Summary of Results

Chloroperoxidase-mediated sulfoxidation of DBT to DBT sulfoxide (DBTSx) in an AOT/isooctane reverse micelle solution has been described and verified by GC/MS. Chloroperoxidase in reverse micelles appears to be a versatile catalyst for sulfoxidation of organic sulfur-containing molecules.

Section 2

MATERIALS AND METHODS

2.1 Materials

Enzyme studies were performed with chloroperoxidase from Caldariomyces fumago. Chloroperoxidase, 2.7 mg/ml in 0.1 M sodium phosphate buffer (pH 4.0), was obtained from Sigma Chemical Co. and refrigerated at 0 °C. DBT (Aldrich Chemical Co.) was evaluated as enzyme substrate. The reverse micelle solutions contained surfactant AOT (0.1 M, di (2-ethylhexyl) sodium sulfosuccinate from Sigma Chemical Co.), organic solvent isooctane (OmniSolv, EM Scientific), and 2.3% or 4.6% (v/v) aqueous phase (potassium phosphate, 0.1 M, pH 2.75). Tetrahydrofuran and acetonitrile, both from VWR Scientific, were used in HPLC analysis of DBT-containing samples. Solid phase extractions were performed on 20H (diol) Bond Elut (500 mg) Bond Elut columns from AnalytiChem Inc. (Van Horne, 1990). Disposable syringe filter units (Rainin Instrument Co., No. 38-159, 0.2 μM pore size, 3 mm diameter Nylon 66 membranes) were used to filter standards and experimental samples of DBT incubations. A Scientific Industries rotator was used for agitation of incubation samples.

2.2 Methods

2.2.1 Quantitative Analysis of DBT Metabolites: HPLC Procedure

Standards for HPLC analysis included: 1) DBT (2 mM) in 0.1 M AOT/isooctane with 4.6% aqueous phase (0.1 M phosphate buffer, pH 2.75); 2)

DBT sulfoxide (0.2 mM) in tetrahydrofuran:acetonitrile:water (23:20:57); and 3) DBT sulfone (0.2 mM) in tetrahydrofuran: acetonitrile:water (23:20:57). DBT sulfoxide and sulfone at ten-fold lower concentrations were not soluble in 0.1 M AOT/isooctane or potassium phosphate buffer (pH 2.75, 0.1 M). Standards and experimental samples were filtered through disposable syringe filter units. Filtrates (5 to 10 µl) were analyzed with a Shimadzu HPLC system containing a C18 resolve (Waters Inc., 5 micron spherical) column. The mobile phase contained tetrahydrofuran:acetonitrile:water (23:20:57) at a programmed rate of 0.8-1.8 ml/min. The specific program was 0-6.5 min, 0.8 ml/min; 6.5-10.min, from 0.8 to 1.8 ml/min; 10-26 min, 1.8 ml/min; 26-30.5 min, from 1.8 to 0.8 ml/min. The wavelength of the detector (SPD-6AV UV-VIS spectrophotometer) was set at 280 nm. The method is a modification of procedures previously used in this project for analysis of DBT metabolites in organic solvents (Wyza et al., 1989).

2.2.2 GC/MS Analysis

GC/MS analyses were performed by Tracer Technologies, Inc., Somerville, MA, using a Hewlett Packard HP5988A mass spectrometer coupled to an HP5890, Series II, gas chromatograph. The instrument was operated at 70 eV in EI mode with on-column sample injection. Signal acquistion and display were carried out on a Teknivent data system. Total ion chromatograms and individual peak fragmentograms at mean peak retention time were recorded for analysis of characteristic ion fragments. For chromatographic separation of DBTSn and DBTSx, a multipurpose, 1.4 micro, thick film, 30 m length, 0.25 mm ID capillary with bonded and cross-linked DB-624 (J&W Scientific) was used. Confirmatory analyses were also carried out with a 0.25 micron, thin film, 30 m length, 0.25 mm ID capillary, with a

bonded and crosslinked SE-54 equivalent (Supelco SPB-5). The former was operated isothermally at 250°C and the latter at 220°C.

2.2.3 Enzyme Assays in Reverse Micelle Solutions

DBT (2 mM) was incubated with chloroperoxidase and H_2O_2 for 2 hours at 23°C in reverse micelle solutions. Solutions were made by adding equal volumes (2.3% of total) of phosphate buffer (0.1 M, pH 2.75) containing 2 μM enzyme and buffer containing H_2O_2 (20 mM) to 0.1 M AOT/isooctane with 2 mM DBT. The solutions were vortexed for 1 minute and then incubated on a rotator. Controls included DBT (2 mM) in AOT/isooctane reverse micelle solution with peroxide but no enzyme. All conditions were tested with triplicate incubations. The filtrate of an enzyme-DBT incubate (0.5 ml) was applied to a 20H (diol) Bond Elut column (500 mg) and eluted with 0.25 ml aliquots of 80:20 chloroform:acetone to minimize AOT in the sample. Eluates 2-4 were collected, the solvent evaporated under N_2, and the residue redissolved in tetrahydrofuran:acetonitrile:water (23:20:57) (0.1 ml) for HPLC analysis. The HPLC effluent from 2.0-2.2 min after injection was collected, evaporated under N_2, and the residue analyzed by GC/MS.

Section 3

RESULTS

3.1 DBT Sulfoxidation by Chloroperoxidase in AOT/Isooctane Reverse Micelle Solutions

DBT was tested as a substrate for chloroperoxidase-mediated sulfoxidation in reverse micelle solution. In this experiment, DBT (2 mM) was incubated with chloroperoxidase and H_2O_2 for 2 hours at 23°C in reverse micelle solutions. A metabolite with the same retention time as DBTSx (1.97-2.1 min, flow rate of 0.8 ml/min) was consistently detected in the enzyme-containing samples but not in controls, consistent with a preliminary study

(Figure 1, 11th Report). An area value at 1.9-2.1 detected for the non-enzyme controls was not a definitive peak and was similar to that for DBT in the reverse micelle solution without enzyme or peroxide. In a subsequent experiment with sequential triplicate addition of enzyme and peroxide (see 11th report for procedure), even greater production of this DBTSx-like metabolite was observed ($p<0.01$, Student's t-test). Approximately 12.5% conversion of DBT to this metabolite was achieved. The results are summarized in Table 1. An additional metabolite with a retention time slightly shorter than that of DBTSn and an area value slightly less than DBTSx was also observed.

3.2 Characterization of Major DBT Metabolite by GC/MS

The chromatograms on DB-624 and individual peak fragmentograms for 0.5 mM standards of DBT (MW184), DBTSn (MW216), and DBTSx (MW200) are presented in Figure 1. For DBTSx, sample A32 showed the characteristic spectrum for the parent ion according to the expected elemental composition. However, a pyrolytic disproportionation reaction may have taken place at the site of on-column injection. As a result, one molecule each of DBT (A31) and DBTSn (A33) was produced from the reaction of two sulfoxides with each other. Otherwise, the presence of DBT in the DBTSx standard cannot be reconciled with its absence in HPLC chromatograms. It is also possible that the DB-624 acted as a reductant in the artifactual conversion of DBTSx to DBT, since this transformation did not take place when SPB-5 was used as the chromatographic liquid phase (Figure 2). As indicated in Figure 2, the chromatogram of DBTSx on SPB-5 indicated a poorly resolved mixture of DBTSx and DBTSn but an absence of DBT.

HPLC eluates with an DBTSx-like retention time (2.0-2.2 min) were collected from three separate enzyme/DBT incubations and analyzed by

GC/MS using DB-624. The critical observation was the presence of a peak at retention time 20.2 min, like the DBTSx standard. The fragmentation pattern of this peak indicated a parent ion of MW200, which verified the identity of this metabolite as DBTSx. The results from all three samples were similar and are illustrated for one sample in Figure 3.

Section 5

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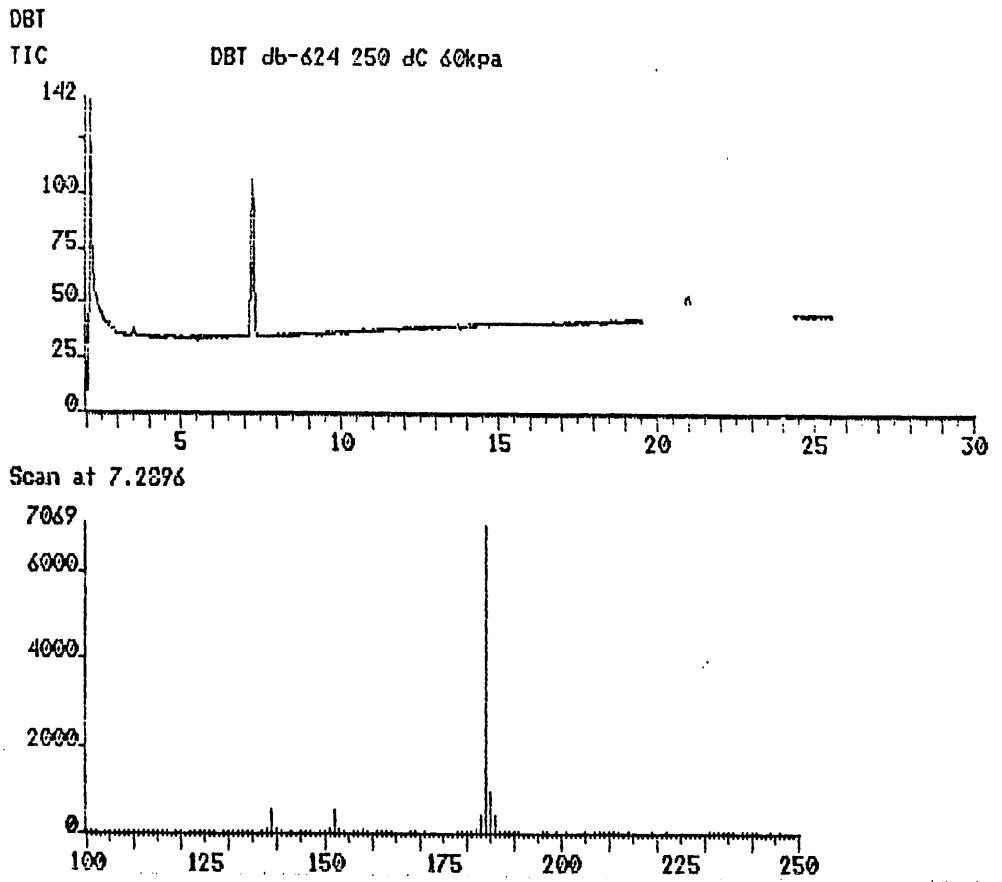
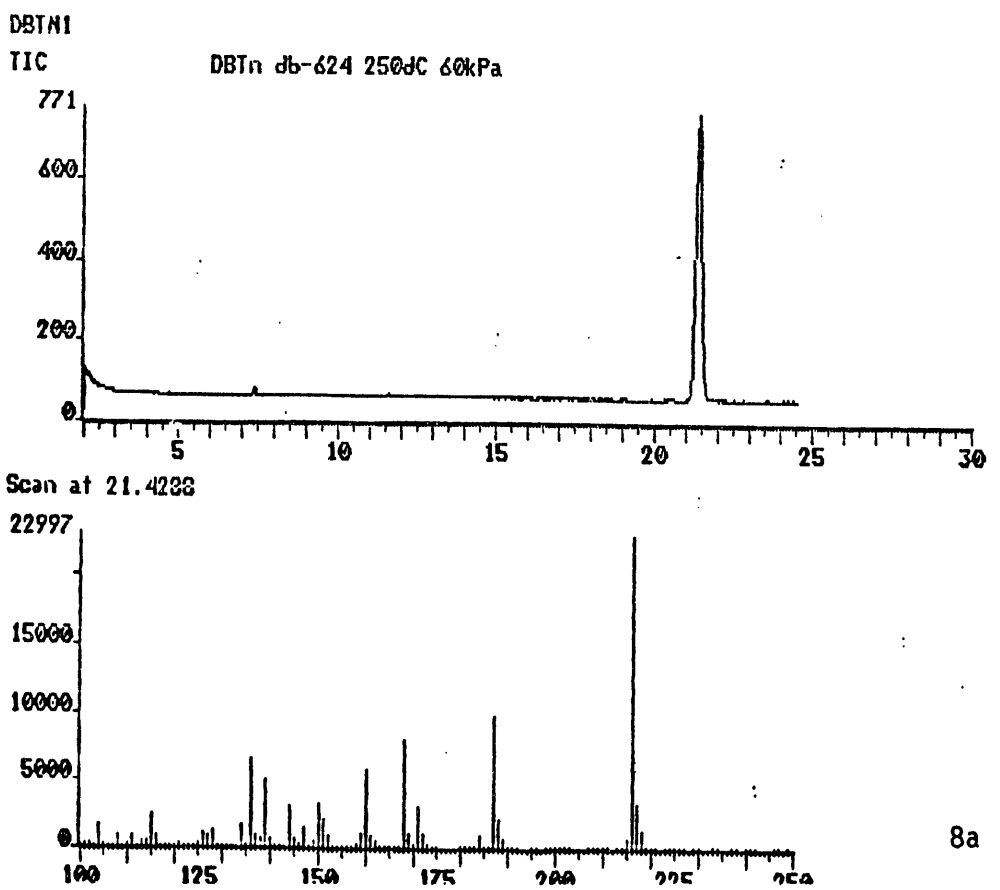
Figure 1: GC/MS Chromatographs (DB-624) and Fragmentograms of DBT, DBTSn, and DBTSx Standards

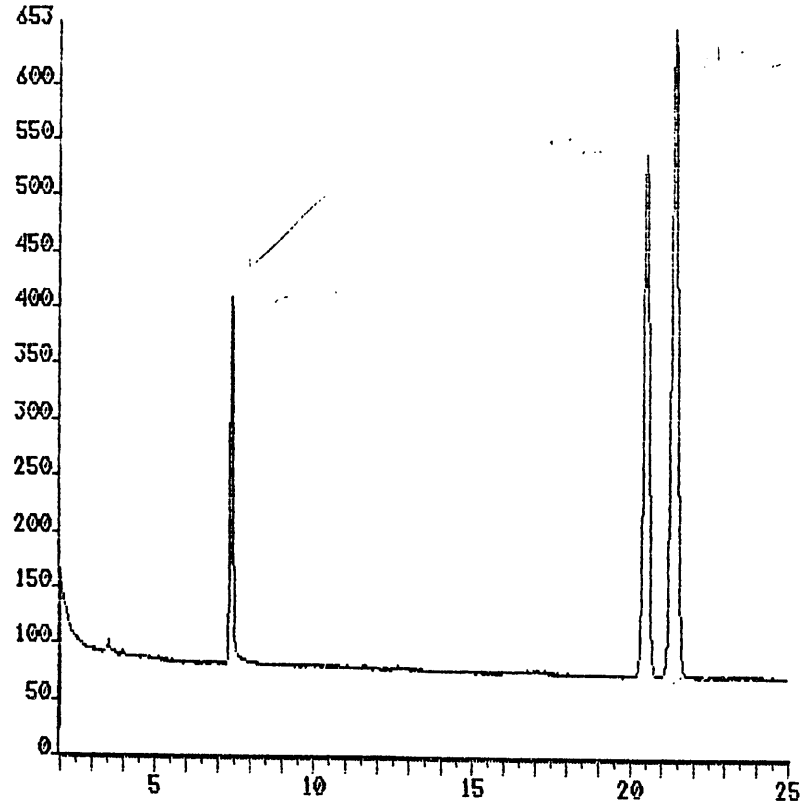


DBT



DBT SULFONE

DBTX1
TIC



DBT SULFOXIDE

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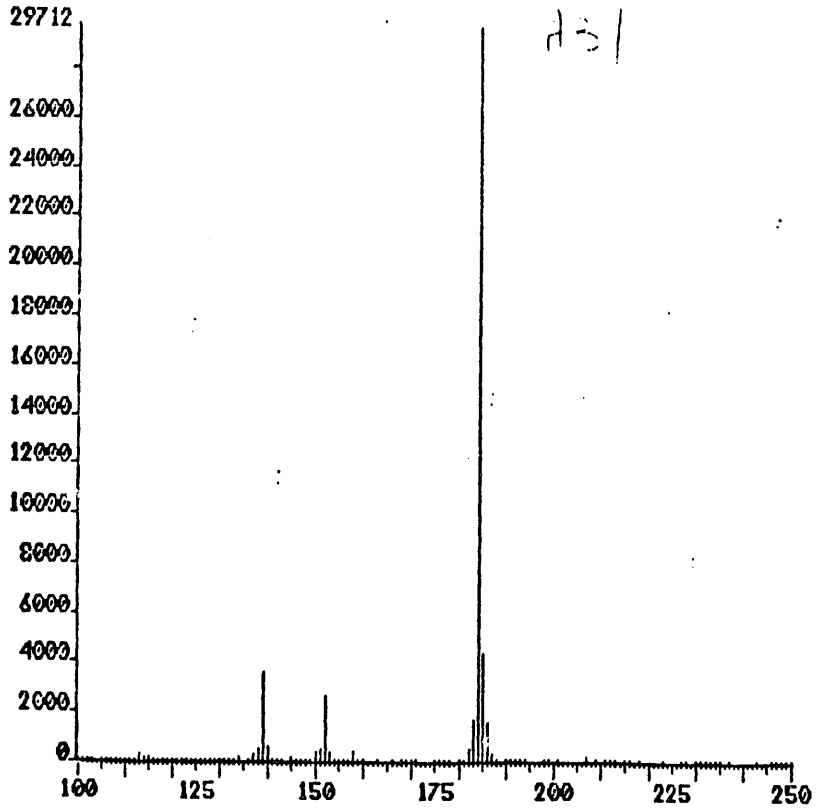

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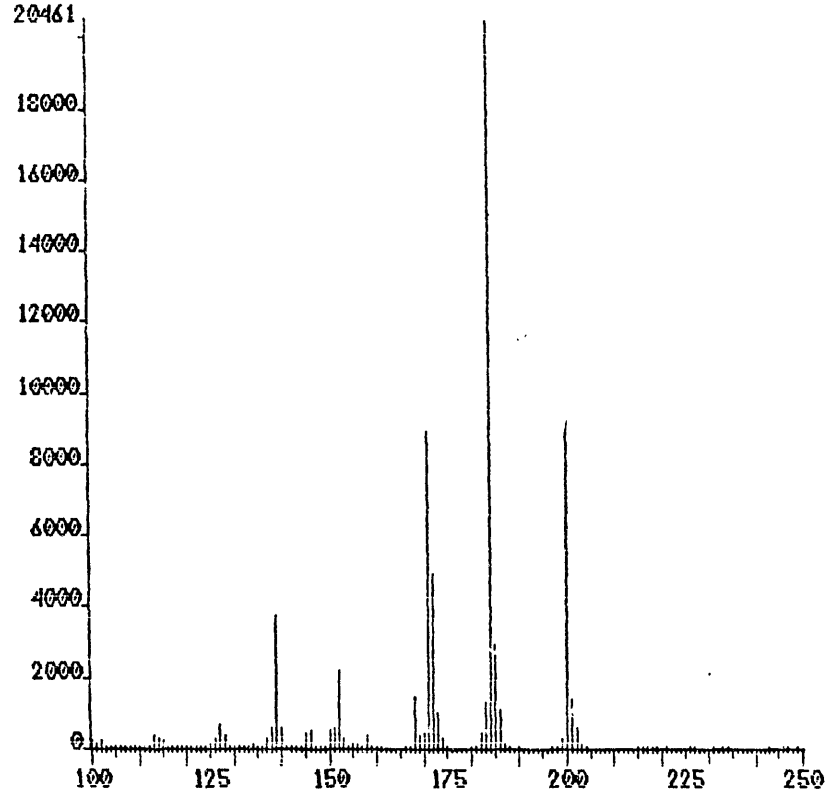

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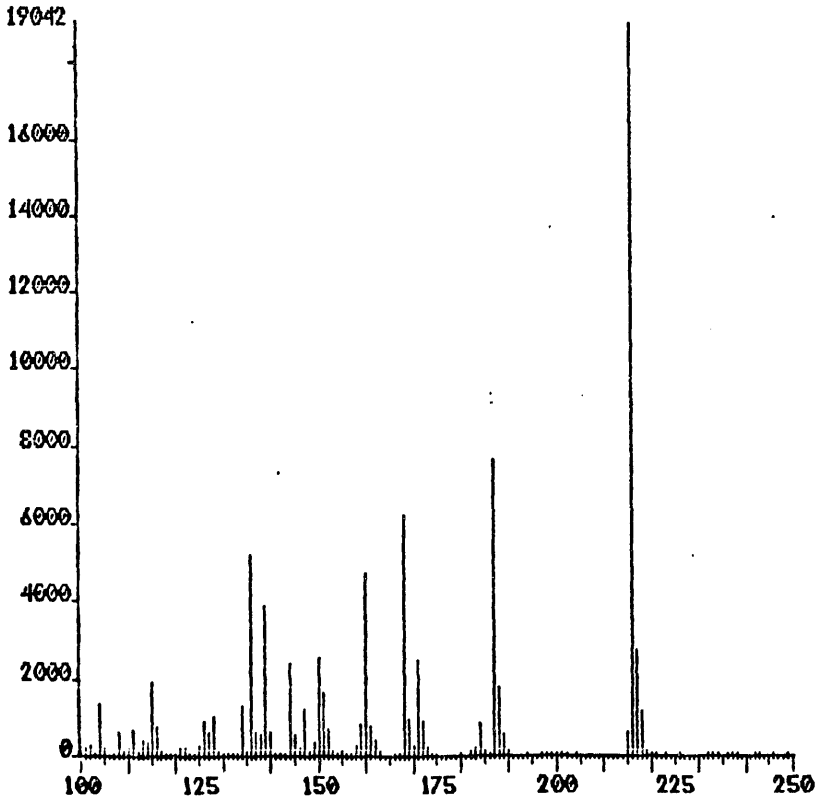

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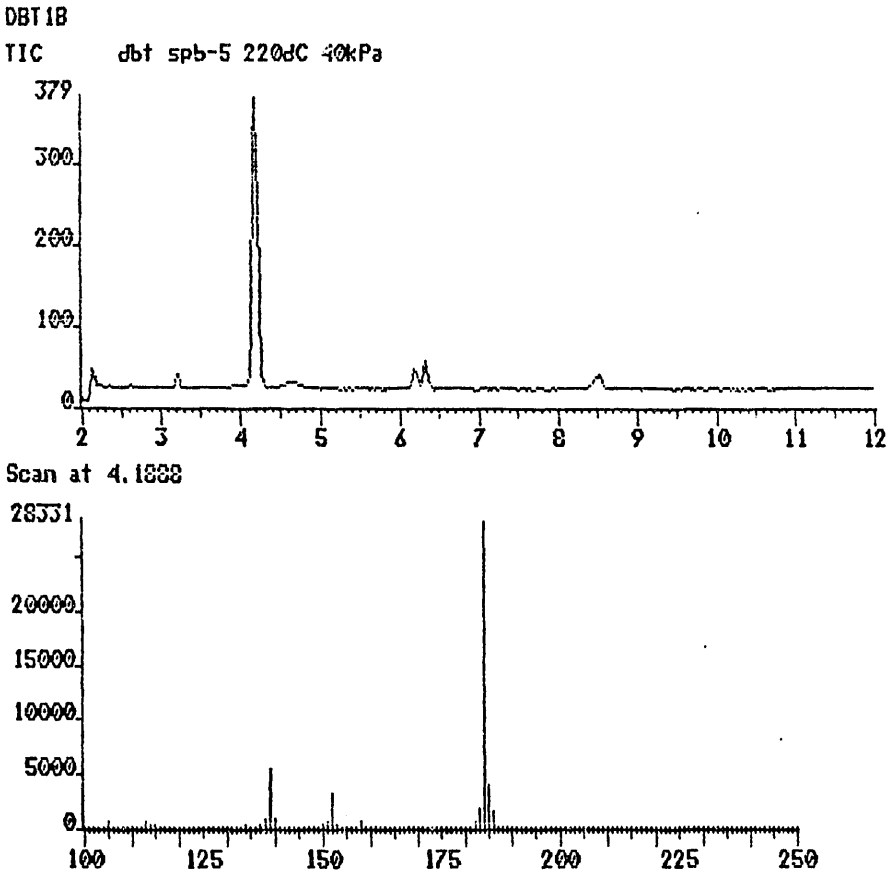
Figure 2: GC/MS Chromatographs (SPB-5) and Fragmentograms of DBT, DBTSn, and DBTSx Standards



DBT

DBTNA

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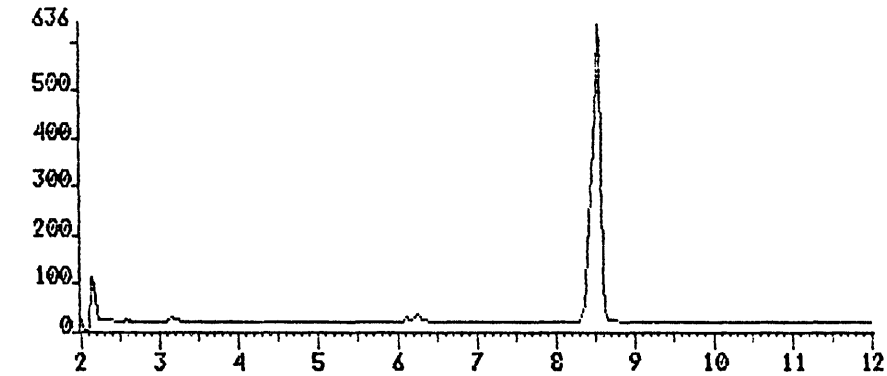

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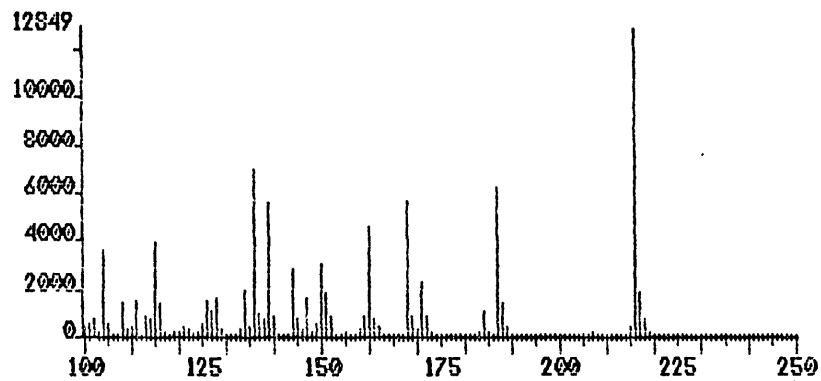

DBT SULFONE

DBTXA

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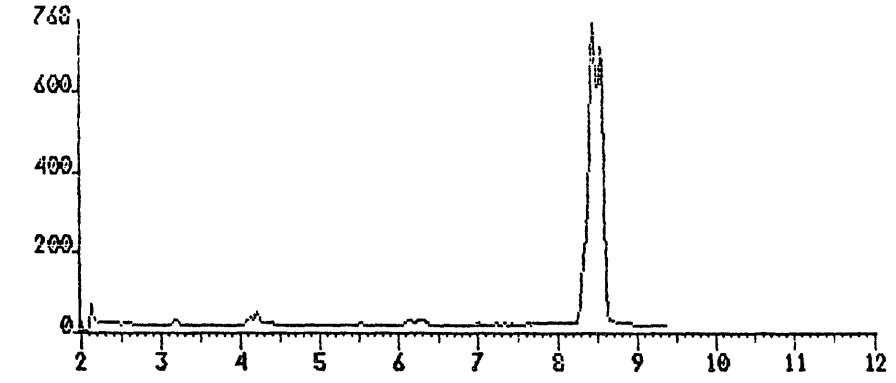

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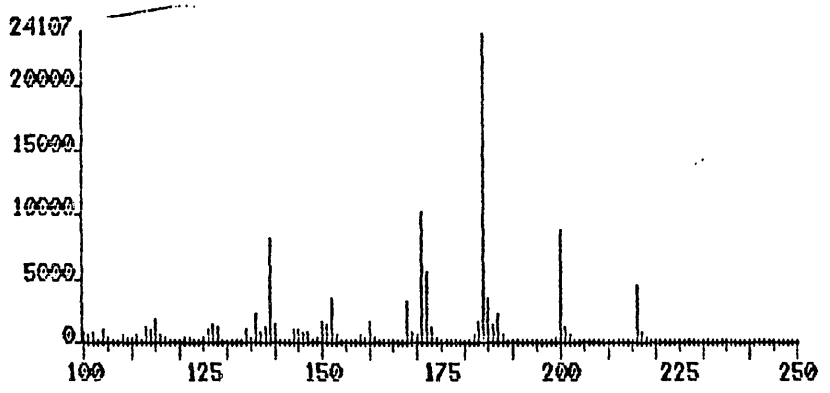
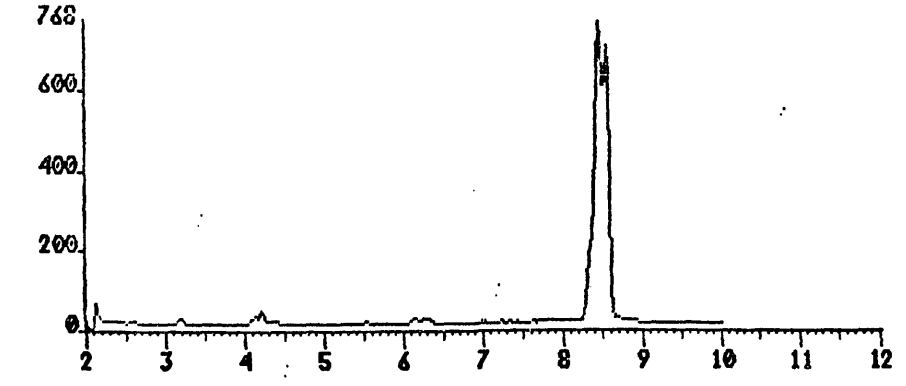


DBT SULFOXIDE

DBTXA

TIC dbtx spb-5 220dC 40kPa



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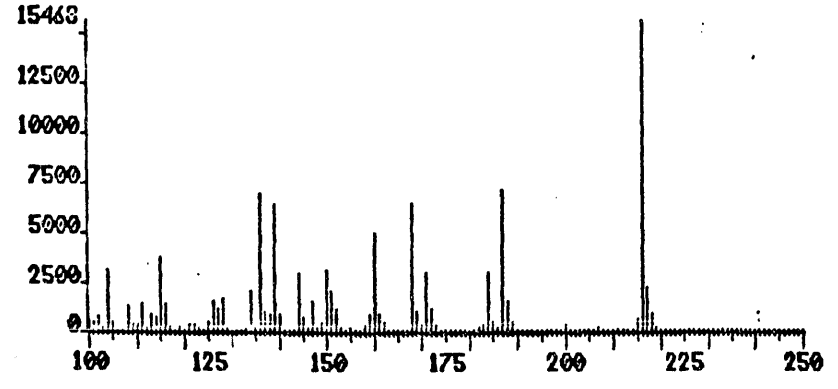

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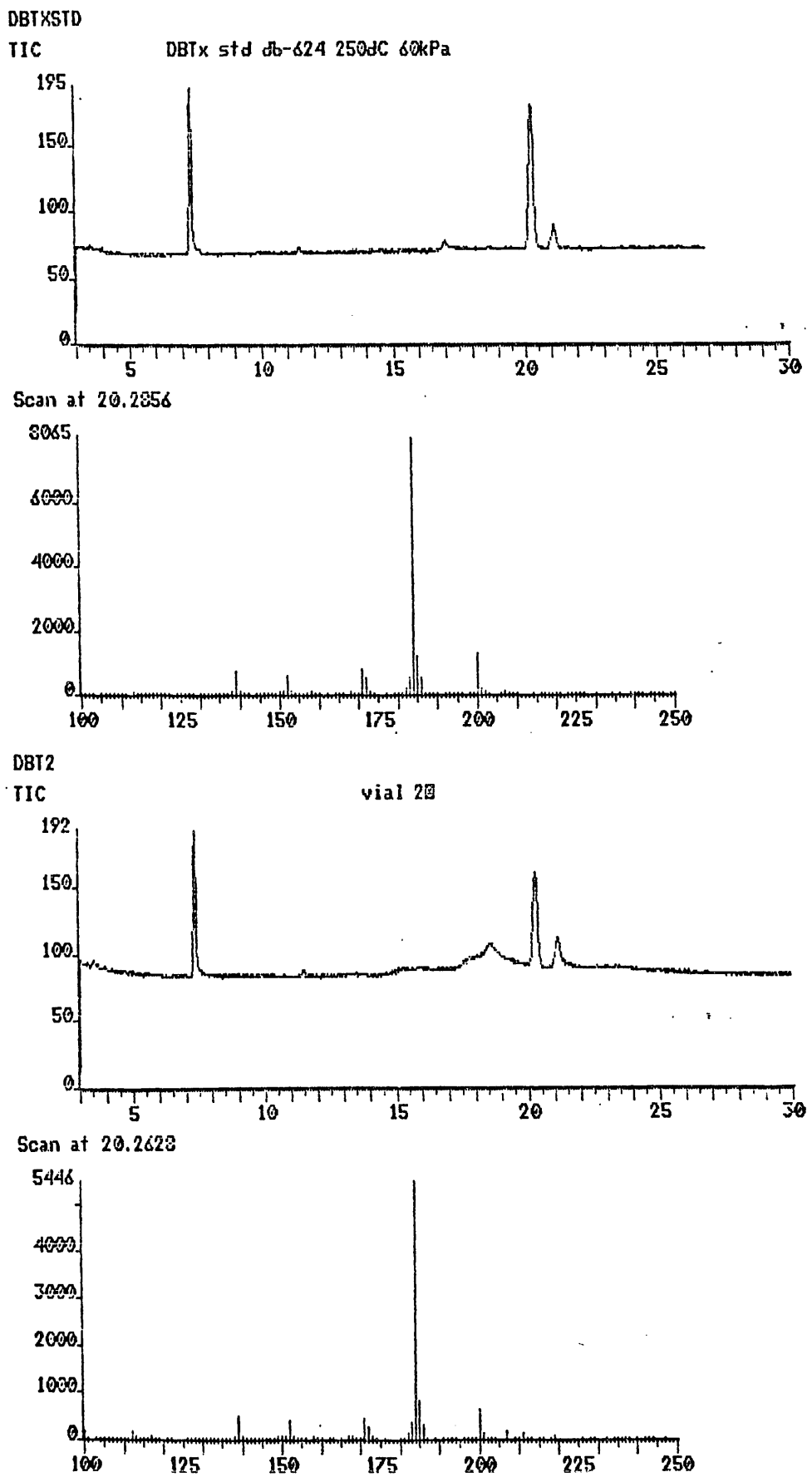
Figure 3: GC/MS Chromatographs (DB-624) and Fragmentograms of DBTSx-like Metabolite (HPLC eluate 2.0-2.2 min) from Chloroperoxidase-Containing Reverse Micelle Solution Incubated with DBT



DBT SULFOXIDE

Experimental Sample

Table 1: Sulfoxidation of DBT in Reverse Micelle Solution by Single and Sequential Addition of Chloroperoxidase and Peroxide.

Method of Enzyme/Peroxide Addition[b]	DBTSx CONCENTRATION[a] (μM) Control	Enzyme
Single	40 ± 16[c]	106 ± 42
	n=3	n=6
Sequential[d]	88 ± 46	253 ± 46
	n=3	n=3

[a] Based on peak area at retention time of 2.0-2.2 min compared to area of DBTSx (0.2 mM, K&K Lab.) in tetrahydrofuran: acetonitrile:water (23:20:57).

[b] Single: Reverse micelle solutions were made by adding phosphate buffer (0.1 M, pH 2.75) containing 2 μM enzyme and H_2O_2 (20 mM), 2.3% of total volume each, to 0.1 M AOT/isooctane with 2 mM DBT. Solutions were incubated for 2 hours at 23°C and analyzed in duplicate. Sequential: Reverse micelle solutions were made by adding chloroperoxidase (2 μM, 1.15% v/v) and then H_2O_2 (20 mM, 1.15% v/v) to 0.1 mM AOT/isooctane with 2 mM DBT. After 0.5 hr enzyme and peroxide were again added (2nd addition). At that time additional AOT/isooctane (1:1 v/v) was added (3rd addition) with enzyme (2.3% v/v) and H_2O_2 (2.3% v/v); samples were incubated for 0.5 hr and then analyzed.

[c] Mean ± SD, n= Number of samples.

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